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Non-covalent imprinting of phosphorous esters

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Abstract

The creation of molecularly imprinted polymers (MIPs) for the recognition of phosphate and phosphonate esters is reported. The single, weak hydrogen-bond acceptor site in these molecules has been targeted using a 1,3-diarylurea functional monomer. Polymers were prepared using either stoichiometric ratios of functional monomer to template or a large excess of the template during imprinting. The recognition properties of the polymers were assessed in the chromatographic mode for their ability to retain the templates and analogous analytes. The results are discussed with regards to the choice and amount of template, polymerisation conditions and the composition of the chromatographic mobile phase.

Keywords: Imprinting; Polymerisable urea; Phosphorous esters; Molecular recognition

1. Introduction

Molecularly imprinted polymers (MIPs) are synthetic, network polymers created by the polymerisation of functional and cross-linking monomers in the presence of a template species [1–5]. The most common and flexible method to prepare MIPs is the non-covalent approach [6]. Here, template species are complexed using a suitable functional monomer and these complexes are then copolymerised with an excess of a cross-linking monomer. Removal of the template molecule reveals binding cavities that are complementary to the template (and analogues thereof) in terms of size, shape and functionality. This technique has proven successful for creating macromolecular receptors for an impressively large variety of molecules, e.g. pharmaceuticals [7,8], environmental pollutants [9,10] and peptides [11,12].

Despite these successes, challenges remain, especially in the preparation of MIPs against templates with little or no functionality amenable to complex formation. This can be attributed to the widespread use of only commercially available functional monomers to target the template species. Such monomers are incapable of providing sufficiently strong interactions with poorly functionalised templates.

Phosphate and phosphonate esters are examples of such compounds. They are widely used as flame-retardants and plasticizers, e.g. triphenyl phosphate (**2**) is commonly used in the fabrication of video display units. Previous studies have shown them to be present in office environments due emission from such electronic equipment [13,14]. They have also been found in environmental waters [15]. Triphenyl phosphate is a contact allergen [16] and has also proven to be a relatively potent inhibitor of human blood monocyte carboxylesterase [17,18]. Of further interest is the fact that organofluorophosphorus nerve agents, such as Soman, Sarin and VX, present similar functionality to the compounds studied here [19].

To our knowledge, there have been no reports on the imprinting of phosphate or phosphonate esters, although hydrolysis products of these species have been successfully imprinted [20–22].

In this paper, we wish to report our attempts to imprint phosphorous esters containing a single hydrogen bond acceptor site, i.e. the P=O functionality, using the urea functional monomer **1**. We have previously described the use of **1** in the imprinting of carboxylates [23]. Similar ureas have been found to co-crystallise with weak hydrogen-bond acceptors

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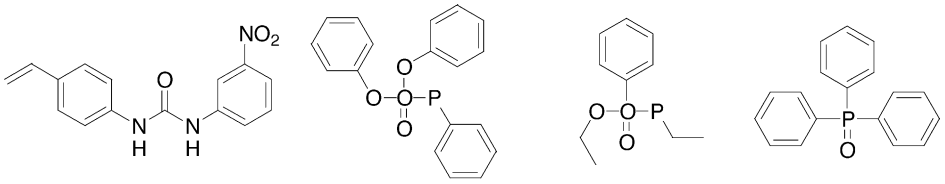

Fig. 1. Chemical structures of the urea-based functional monomer and the templates/analytes used in this study.

such as ketones and cyclic ethers [24]. We wished to investigate whether such interactions would persist in the solution state and prove useful for the imprinting of the phosphorous esters triphenyl phosphate (**2**) and diethylphenyl phosphate (**3**) (Fig. 1).

2. Experimental

2.1. Materials

Functional monomer **1** was prepared as described previously [23]. The templates **2** and **3**, and the analyte triphenyl phosphine oxide (**4**) were purchased from Aldrich (Deisenhofen, Germany) and were used as received. Ethyleneglycol dimethacrylate (EDMA) and methyl methacrylate (MMA) were obtained from Aldrich (Deisenhofen, Germany) and purified prior to use. The monomers were washed sequentially with 10% aqueous NaOH, water and brine. After drying over $MgSO_4$, the pure reagents were obtained via distillation under reduced pressure. Anhydrous THF was obtained from Fluka (Deisenhofen, Germany). Acetonitrile (ACN), chloroform and heptane (all HPLC grade) were from Merck (Darmstadt, Germany). The free radical initiator 2,2′-azobis(2,4-dimethylvaleronitrile) (ABDV) was obtained from Wako (Neuss, Germany) and was used as received. CD_3CN and THF-d_8 were obtained from Deuterio GmbH (Kastellaun, Germany).

2.2. ^1H NMR titrations

Titrations were performed in CD_3CN or THF-d_6. An increasing amount of guest molecule (**2**–**4**) was titrated into a constant amount of host monomer. The monomer concentration was 5 mM and the amounts of added guest were 0, 0.5, 1, 2, 3, 4, 5, 7.5 and 10 equiv., i.e. 0–50 mM, respectively. The complexation-induced shifts (CIS) of the urea protons were monitored and titration curves were constructed. The raw titration data were fitted to a 1:1 binding isotherm [25] and the association constants were obtained by non-linear regression of the isotherms (using Microcal^{TM} Origin 5.0).

2.3. Polymer preparation

Two series of MIPs were prepared using either **2** or **3** as template and **1** as the functional monomer. The imprinted polymers P2S, P3S were prepared using a stoichiometric ratio of template to **1**, while P2E and P3E were prepared using a 10-fold excess of template to **1**. Control, non-imprinted polymers, PNS and PNE were prepared in the same manner as the corresponding MIPs, but without the addition of a template species. Further control polymers for the excess template MIPs, P2EC and P3EC, were prepared by substituting MMA for **1** as the functional monomer. The composition of each pre-polymerisation mixture is shown in Table 1. The solutions were each placed in borosilicate tubes, cooled (0 °C) and then degassed by bubbling N_2 through them for 10 min. All polymerisations were initiated thermally at 40 °C, using ABDV as the initiator, and allowed to continue for 24 h. After polymerisation, the monolithic polymers were broken into small pieces and extracted with methanol in a Soxhlet apparatus for 24 h. The washed polymers were then crushed and sieved and particles of size 25–36 μm were collected. These particles were repeatedly sedimented in MeOH/H_2O (80/20 v/v) to remove fine material and then slurry packed into stainless steel HPLC columns (125 mm × 4.6 mm i.d. or 33 mm × 4 mm i.d.) for evaluation of their recognition properties in the chromatographic mode. Column packing was

Table 1
Pre-polymerisation solution compositions

Polymer	Template (g)	Functional monomer (g) (1 mmol)	EDMA (g) (20 mmol)	ABDV (mg)	Solvent (mL)
P2S	**2**, 0.326 (1 mmol)	**1**, 0.283	3.964	42.5	THF (5.6)
P3S	**3**, 0.214 (1 mmol)	**1**, 0.283	3.964	42.5	THF (5.6)
PNS	–	**1**, 0.283	3.964	42.5	THF (5.6)
P2E	**2**, 3.26 (10 mmol)	**1**, 0.283	3.964	42.5	THF (2.8), ACN (2.8)
P3E	**3**, 2.14 (10 mmol)	**1**, 0.283	3.964	42.5	THF (2.8), ACN (2.8)
PNE	–	**1**, 0.283	3.964	42.5	THF (2.8), ACN (2.8)
P2EC	**2**, 3.26 (10 mmol)	MMA, 0.100	3.964	40.6	THF (2.8), ACN (2.8)
P3EC	**3**, 2.14 (10 mmol)	MMA, 0.100	3.964	40.6	THF (2.8), ACN (2.8)

achieved using a compressed-gas slurry packing apparatus at a maximum pressure of 300 mbar, using MeOH/H_2O (80/20 v/v) as the pushing solvent.

2.4. Chromatographic experiments

HPLC evaluation of the prepared polymers was performed using an HP1100 system, equipped with a binary pump and a variable wavelength UV detector, coupled to an HP workstation. For all experiments, the analyte solution injection volume was 10 μL and a flow rate was 1 mL/min was used. A minimum of four separate injections was made for each analyte on each column. A non-polar and polar mobile phase were used in the evaluation of the polymers, namely chloroform/heptane (1/1 v/v) and 100% ACN. Retention factors (k) were calculated as:

$$k = \frac{t_R - t_0}{t_0} \quad (1)$$

where t_R is the retention time of the analyte and t_0 the retention time of the void volume marker (either acetone or toluene). Imprinting factors (IF) were calculated as:

$$IF = \frac{k_{imp}}{k_{non\text{-}imp}} \quad (2)$$

3. Results and discussion

Prior to preparing the imprinted polymers, the ability of **1** to associate with the templates in solution was studied via ^{1}H NMR titrations. Due to the insolubility of **1** in less polar solvents, such as $CDCl_3$, CCl_4 or deuterated alkanes, these experiments were initially performed in CD_3CN. Addition of up to 10 equivalents of either **2** or **3** caused no noticeable change in the chemical shifts of the urea protons. In addition to the templates, we also investigated the solution interactions of **1** with **4**. In this case, addition of 10 equivalents of **4** to the solution of **1** produced a CIS of 1.25 ppm. Fitting of the raw titration data to a 1:1 binding isotherm gave $K_a = 80\ M^{-1}$ for the **1**:**4** association. A similar titration, performed in THF-d_8, led to CIS of only 0.09 ppm for the urea protons; no association constant could be determined from this experiment. However, our observations agree with previous IR studies, performed in chloroform or CCl_4 and using phenolic compounds as the hydrogen bond donor, which showed that the order of hydrogen-bond acceptance strength (Lewis basicity) for molecules of this type is phosphine oxides ≫ phosphonate esters > phosphate esters [26–28]. Further, the fact that neither **2** nor **3** causes a CIS of the urea protons implies that they are poorer hydrogen-bond acceptors than the solvent (CD_3CN).

Despite the lack of noticeable interactions between **1** and either template (in CD_3CN), we proceeded to prepare the 'stoichiometric' MIPs, P2S and P3S, along with the control polymer PNS, using THF as the polymerisation solvent. An imprinted polymer was not prepared against **4** due to its insolubility at the desired concentrations for polymerisation. While the use of THF as solvent should lead to weaker interactions between the templates and **1** than would the use of acetonitrile, the components of the pre-polymerisation mixtures were not sufficiently soluble in acetonitrile. After template removal, the polymers were crushed, sized and packed into columns for evaluation in the chromatographic mode. Both templates were used as analytes, along with **4**, and a non-polar mobile phase, heptane/chloroform (1/1 v/v), was used so as to favour hydrogen-bond interactions between the analytes and polymers. The results are shown in Fig. 2 and Table 2.

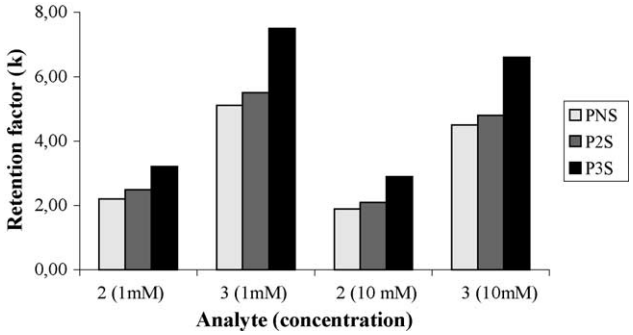

Fig. 2. Retention factors for **2** and **3** on the 'stoichiometric' imprinted and non-imprinted polymers. Conditions: mobile phase = heptane/chloroform (1/1 v/v) injection volume = 10 μL, column dimensions = 125 mm × 4.6 mm i.d. Analyte detection was performed at λ = 260 nm. The void volume marker was acetone.

On the non-imprinted polymer, PNS, the order of retention is **4** ≫ **3** > **2**. This is not surprising and can be explained from relative order of Lewis basicity of these molecules. Turning to P2S, while the retention order is the same, a small but definite imprinting effect is observed. The imprinting factor (IF) is highest at the lowest injected concentration (IF = 1.2 at 0.1 mM) and falls off at higher sample loads. This suggests that the imprinted sites in P2S are non-uniform in nature, in accordance with the majority of non-covalent MIPs. In the case of P3S, the polymer is able to retain its template more strongly than either the PNS or P2S, leading to an IF = 1.5. This suggests that **1** possesses some ability to bind to **3** under the conditions employed during the polymerisation and thus create binding sites that are better defined than those in P2S. Despite this, **4** is still far more strongly re-

Table 2
Retention factors for **2**–**4** on the 'stoichiometric' imprinted and non-imprinted polymers

Analyte (mM)	k(PNS)	k(P2S)	IF	k(P3S)	IF
2 (0.1)	2.2	2.7	1.2	2.7	
2 (1)	2.2	2.5	1.1	2.9	
2 (10)	1.9	2.1	1.1	2.6	
3 (1)	5.1	5.5		7.5	1.5
3 (10)	4.5	4.8		6.6	1.5
4 (1)	32.3	35.9		–	
4 (10)	28.9	31.9		40.7	

For conditions, see legend in Fig. 2.

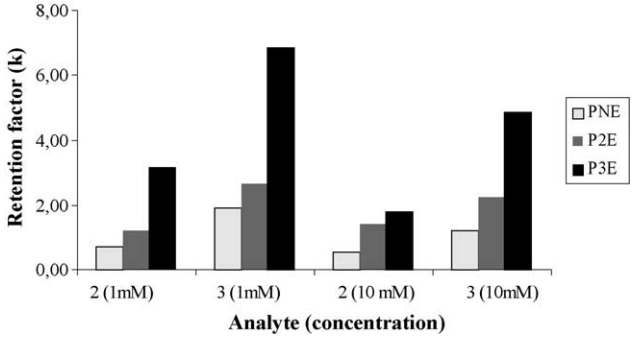

Fig. 3. Retention factors for **2** and **3** on the 'excess template' imprinted and non-imprinted polymers. Conditions: mobile phase = chloroform/heptane (1/1 v/v), injection volume = 10 μL, column dimensions = 33 mm × 4 mm i.d. Analyte detection was performed at λ = 260 nm. The void volume marker was toluene.

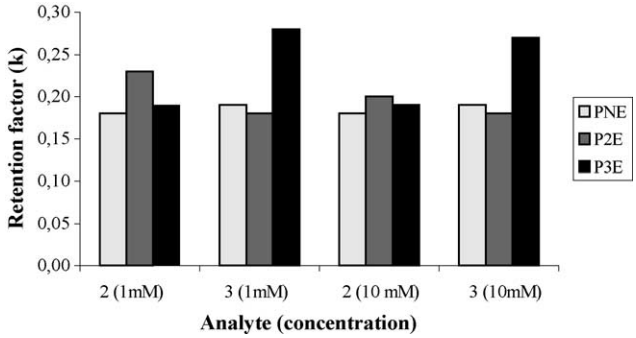

Fig. 4. Retention factors for **2** and **3** on the 'excess template' imprinted and non-imprinted polymers. Conditions: mobile phase = acetonitrile, injection volume = 10 μL, column dimensions = 125 mm × 4.6 mm i.d. Analyte detection was performed at λ = 260 nm. The void volume marker was acetone.

tained than **3**. It is interesting to note that the ratio of the retention factors for these analytes on all three polymers is k(**2**):k(**3**):k(**4**) ≈ 1:2.3:15. This ratio is similar to the ratio of association constants found for the interactions between these compounds and phenol [26–28].

Due to the limited success of the imprinting protocol described above, we pursued a second approach. MIPs P2E and P3E were prepared using a 10-fold excess of the template molecules with respect to **1** [29], with the aim of pushing the respective monomer–template interaction equilibria to favour complex formation. This process was further aided by a change of the polymerisation solution to a 1:1 mixture of ACN and THF. The presence of the excess templates appears to aid in the dissolution of the components of the pre-polymerisation solution, with **3** being especially good in this regard. As well as preparing the non-imprinted polymer PNE, two further control polymers were synthesised, namely P2EC and P3EC. Here, the large excess of templates was retained, but MMA was used as the functional monomer in place of **1**. No significant interactions are expected between MMA and either of the templates. These control polymers were prepared in order to show that it is the interaction of **1** with the respective templates that gives rise to imprinting and not simply the presence of the template (in large excess) during the polymerisation. Using chloroform/heptane (1/1 v/v) as the mobile phase, all materials were assessed for their ability to retain **2**–**4** (Fig. 3 and Table 3).

Table 3
Retention factors for **2**–**4** on the 'excess template' imprinted, non-imprinted and control polymers

Analyte (mM)	k(PNE)	k(P2EC)	k(P3EC)	k(P2E)	IF	k(P3E)	IF
2 (1)	0.7	0	0.1	1.2	1.7	3.1	
2 (10)	0.5	0	0.1	1.4	2.8	1.8	
3 (1)	1.9	0	0.2	2.6		6.8	3.6
3 (10)	1.2	0	0.2	2.2		4.8	4.0
4 (1)	10.1	0.04	0.7	15.5		29.2	
4 (10)	6.3	0	0.3	14.9		31.9	

For conditions, see legend in Fig. 3.

Comparing the retention behaviour of the analytes on P2E, P3E and PNE, we see that there is more definite evidence of imprinting than observed for the stoichiometrically imprinted polymers. Thus, for **2** on P2E, the IFs are 1.7 (1 mM) and 2.8 (10 mM), respectively, while for **3** on P3E, the IFs are even more impressive with values of 3.6 (1 mM) and 4.0 (10 mM), respectively. However, while the imprinting effect is demonstrated, the retention order of the analytes again reflects the hydrogen-bonding ability of the analytes, i.e. k(**4**) > k(**3**) > k(**2**). Thus, in our present system and under these evaluation conditions, the act of imprinting is not sufficient to overturn the retention order caused by the primary polymer–analyte interaction, i.e. that between the urea and the P=O functions.

That the proposed interactions are indeed responsible for the imprinting and retention order is shown by the retention behaviour of the analytes on the control polymers, P2EC and P3EC. All analytes were eluted quickly from both columns, with k-values in the range 0–0.7 and showing no discernible trend. Thus, the presence of the excess template alone does not give rise to the imprinting in these systems and implies that the interaction of the analytes with the residues of **1** in the polymers is essential in the retention/recognition process in our materials.

The 'excess-template' polymers were then assessed using a more polar mobile phase, namely acetonitrile; the results are shown in Fig. 4 and Table 4. There is a dramatic decrease in retention on changing to the more polar mobile phase, due

Table 4
Retention factors for **2**–**4** on the 'excess template' imprinted, non-imprinted and control polymers

Analyte (mM)	k(PNE)	k(P2EC)	k(P3EC)	k(P2E)	IF	k(P3E)	IF
2 (1)	0.18	0.15	0.12	0.23	1.3	0.19	
2 (10)	0.18	0.15	0.11	0.20	1.1	0.19	
3 (1)	0.19	0.02	0.03	0.18		0.28	1.5
3 (10)	0.19	0.02	0.03	0.18		0.27	1.4
4 (1)	1.55	0.18	0.22	1.59		2.25	
4 (10)	1.44	0.17	0.18	1.40		1.97	

For conditions, see legend in Fig. 4.

to the substantial weakening of the polymer–analyte interactions in this solvent (cf. solution NMR experiments). While the retention factors are low, they are the result of multiple (minimum six) injections at each analyte concentration and are, therefore, reliable.

On PNE, the retention order of the analytes is as before, though there is now less difference between the retention of **2** and **3**. Further, the retention of **4** is also reduced, thus demonstrating the effect of increasing the polarity of the mobile phase. Comparing the behaviour of the analytes on P2E and PNE, we again find evidence of imprinting, with IF values of 1.3 (**2**, 1 mM) and 1.1 (**2**, 10 mM), respectively. More interestingly, P2E retains its template **2** longer than analyte **3** under these conditions, with retention times of 1.9 versus 1.8 min, respectively, as shown in Fig. 5A. While this difference is small, the expected order of retention, based on the Lewis basicity of the analytes, has been overturned and presents further evidence of successful imprinting. It is plausible to suggest that the polar mobile phase, while causing a general reduction in binding to P2E, suppresses non-specific binding of the analytes to a greater extent than it does binding to the imprinted sites. On P3E, an imprinting effect is again observed, with IFs of 1.5 (**3**, 1 mM) and 1.4 (**3**, 1 mM), respectively, while the retention of **2** on this polymer is the same as on the non-imprinted polymer PNE. As shown in the chromatograms in Fig. 5A and B, the peak shapes for the analytes on their imprinted polymers show signs of broadening and tailing (when compared to the peak shapes on the non-complimentary polymer), a feature common to MIP-based stationary phases.

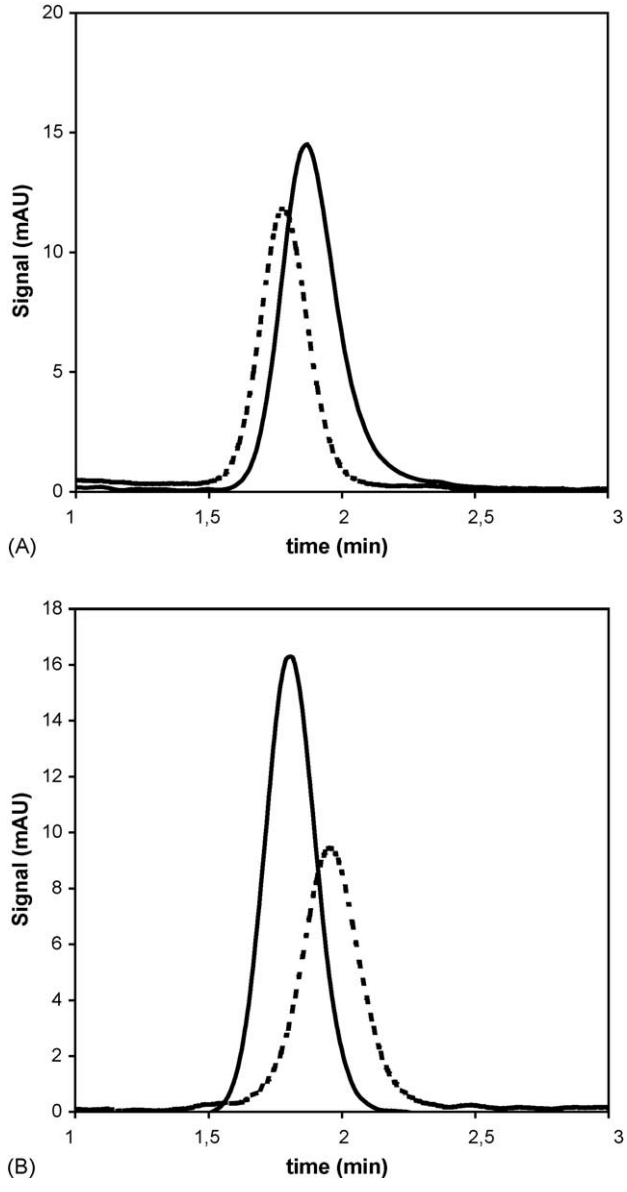

Fig. 5. Chromatograms of **2** (solid lines) and **3** (dashed lines) on (A) P2E and (B) P3E. For conditions, see Fig. 4.

Analyte **4** is still the most retained analyte on all the polymers, again in the order k(PNE) < k(P2E) < k(P3E). Retention of the analytes on P2EC and P3EC is negligible ($k < 0.1$) and no trend is discernible, once again demonstrating that the main driving force for retention of the analytes in our systems is the interaction between the analytes and the polymeric urea moieties.

4. Conclusions

The successful non-covalent molecular imprinting of phosphate and phosphonate esters using a urea-based functional monomer has been demonstrated. When stoichiometric amounts of template and functional monomer are used to create the MIPs, the imprinting effect is present, but weak. An alternative approach, using an excess of template compared with the functional monomer, leads to higher imprinting effects, in both non-polar and polar mobile phases. The retention behaviour of the analytes in the non-polar mobile phase can be explained in terms of the Lewis basicity of the analytes, with retention following the order **4** > **3** > **2**. However, the retention order of the phosphate ester **2** and a phosphonate ester **3** are reversed on the polymer imprinted using an excess of **2**, when using a polar mobile phase.

Since chromatographic evaluation of MIPs tend to probe sites of lower affinity, it is likely that lower load evaluations, for example in sensor formats, will lead to enhanced effects. We are currently continuing our investigations on these systems, using urea-based monomers that bind more strongly than **1** and that are more lipophilic, thus enabling the use of less polar solvents in the preparation of the MIPs.

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